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STUDIES ON THE ROLE OF CALPROTECTIN AS A MEDIATOR OF IRON-INDUCED INFLAMMATION WITH IMPLICATIONS IN PARKINSON'S DISEASE

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2019

Acknowledgments

É impossível deixar de agradecer, no final desta tão importante etapa académica, a todos aqueles que me acompanharam ao longo destes dois anos e que de alguma forma, contribuíram e permitiram a realização desta tese.

Começo assim por agradecer aos meus orientadores: Doutor Cláudio Gomes e Doutora Raffaela Gozzelino, os principais fundadores deste projeto. Ao Prof. Cláudio, agradeço a enorme disponibilidade, e todo o tempo que dedicou para me ajudar nos momentos em que foi necessário, pela paciência e por todos os ensinamentos. À Raffi um enorme obrigada pelo apoio incondicional, por todas as palavras de incentivo, pelo exemplo de força e por puxar por mim, não só a nível académico, mas também artístico. Tenho por ambos um enorme respeito e admiração, e não posso deixar de agradecer por terem encarnado, realmente o papel que julgo ser o de um orientador e por me ajudarem sempre em tudo que lhe foi possível.

Quero agradecer principalmente a toda a minha família, especialmente aos meus pais, Assédio e Fátima e irmãos, Juliana e Bruno, por todo o apoio que me têm dado desde o início do meu percurso académico bem como em toda a minha vida. Por todo o interesse que demonstram no que faço, por saberem que me faz feliz, não importando o que seja! Agradeço do fundo coração!

Não esqueço todos os meus colegas de laboratório: Carina, Joanas, Mariana, Romina e Guilherme um obrigada pelo apoio, pela amizade, por tudo o que me ensinaram, pelas caças ao tesouro e por todas as gargalhadas; Maria e Patrícia, amigas que guardo sempre; um especial obrigada à Ana e à Illy, por serem tão boa companhia, me ajudarem tanto e me apoiarem sempre, em todos os níveis.

À FCUL e ao CEDOC por todos os amigos que me trouxe, os quais sei que ficaram para sempre. E às amigas de Macedo.

À música, minha eterna companheira. E ao meu destino.

Obrigada!

Summary

The accumulation of alpha-synuclein (α-syn) aggregates in the brain, known as Lewy bodies, has been associated with the development of Parkinson's disease (PD), a neurodegenerative disorder characterized by the loss of dopaminergic neurons in the *substantia nigra*. In the recent years, the hypothesis that α-syn might start aggregating in the gut and then transported to the brain, through the direct communication existing between these two organs and mediated by the vagal nerve and efferent neurons, is taken more and more into consideration. This leads to the question of whether PD originates in the gut. Indeed, this compartment shares with the brain several features that characterize the pathogenesis of PD, such as inflammation, barriers disruption and iron accumulation. Increasing concentration of this metal has been observed in both the gut and the brain of mice with advancing age and/or induced with a pharmacological model of PD.

The cross-talk between iron metabolism and inflammation allowed hypothesizing that an enhanced accumulation of this metal in the gut could influence the severity of gut inflammation, assessed by the expression of calprotectin (CP). This is one of the most abundant pro-inflammatory cytokines in the gut, a heterodimer formed by S100A8 and S100A9 subunits. The increased level of calprotectin in conditions characterized by chronic gut inflammation led this protein to become a prognostic and diagnostic marker for gut inflammatory syndromes. Interestingly, the ability of CP to bind Iron allows hypothesizing that CP might act as iron scavenger. This notion is supported by the observation that this protein binds only the reduced and most reactive form of iron, i.e. Fe(II). The anaerobic environment of the gut impedes the oxidation of Fe(II) to Fe(III), thus turning this a perfect compartment for CP to work. Since Fe(II) also favours the aggregation of α-syn, the neutralization of iron reactivity by CP, might prevent and/or delay the development of PD.

Our results demonstrate that aging leads to increased iron concentration in the gut, inflammation and increased intestinal permeability. Consequently, inflammation leads to increased release of CP, which acts as an iron scavenger, binding Fe in its reactive form. Its expression increases in the presence of α-syn, probably synergistically, indicating a relationship between the two proteins. These are promising results supporting the ability of CP as a biomarker of iron-induced intestinal inflammation in the development of Parkinson's disease as a preventive method for the development of PD.

Keywords: Calprotectin, inflammation, iron, aging and alpha-synuclein.

Resumo

A doença de Parkinson (DP) é a segunda doença neurodegenerativa mais comum, seguida pela doença de Alzheimer, e a sua prevalência aumenta significativamente com o aumento da idade. Os elementos que definem a DP são a presença de tremores, rigidez muscular e lentidão nos movimentos. No entanto, sintomas gastrointestinais também são comuns. Esta doença é caracterizada pela perda progressiva dos neurónios dopaminérgicos, presentes na *substantia nigra* do cérebro, a qual leva à formação de *Lewy bodies*, maioritariamente compostos por agregados de alfa-sinucleína (α-syn).

Nos últimos anos a hipótese de que α-syn pode começar a sua agregação no intestino e só depois ser transportada para o cérebro, através de uma comunicação direta que existe entre os dois órgãos e mediada pelo nervo vago e pelos neurónios eferentes, tem sido cada vez mais considerada. Esta afirmação leva-nos a seguinte questão: será que a DP tem origem no intestino? É sabido que este órgão possui, em comum com o cérebro, várias características da DP, assim como inflamação, disrupção da barreira intestinal e acumulação de ferro. O aumento da concentração deste metal tem sido observado em ambos os órgãos, cérebro e intestino, em ratinhos de idade avançada e/ou induzidos com um modelo farmacológico da DP.

O *cross-talk* entre o metabolismo do ferro e a inflamação levantou a hipótese de que a acumulação deste metal no intestino pode influenciar a gravidade da inflamação intestinal, sendo esta avaliada pela expressão de calprotectina (CP). CP é um heterodímero de duas proteínas, S100A8 e S100A9 e é essencialmente encontrada nos grânulos citoplasmáticos dos neutrófilos. A acumulação de ferro no intestino com o avanço da idade provoca a ativação da resposta imune que desencadeia a libertação da CP em circulação. Em doenças inflamatórias (DII), como a Doença de Crohn e retocolite ulcerativa, as concentrações de CP observadas nas fezes são elevadas.

Na presença de cálcio, esta proteína possui a aptidão de sequestrar metais de transição, tais como o ferro. A capacidade da CP de se ligar ao ferro vem de encontro com a hipótese de que a CP pode atuar como eliminador de ferro. Noção esta suportada pelo facto desta proteína se ligar apenas à forma reduzida e mais reativa de ferro, i.e., Fe(II). O ambiente anaeróbico do intestino impede a oxidação do Fe(II) em Fe(III), tornando-o num compartimento perfeito para a ação da CP. Recentemente, foi relatado que a CP pode ligar o Fe(II) e bloquear a captação microbiana de Fe(II), privando as bactérias desse nutriente essencial. Esta informação apadrinha a ideia de que a CP pode influenciar a especiação redox de Fe, desempenhando um papel fisiológico na homeostase do ferro entre patógeno-hospedeiro. Como o Fe(II) também favorece a agregação de α-syn, a neutralização da reatividade do ferro pela CP pode impedir e/ou retardar o desenvolvimento da DP.

Esta dissertação visou testar a conexão entre eventos inflamatórios no intestino, os níveis de CP e o desenvolvimento da DP. Sendo o envelhecimento o principal fator de risco para doenças neurodegenerativas, como a DP, este pode ser considerado um distúrbio inflamatório crônico subagudo. Aqui, demonstramos um aumento de inflamação durante o envelhecimento, observando o aumento dos níveis de interleucina 6 (IL-6), uma citocina pro-inflamatória envolvida na resposta inflamatória aguda, em ratinhos mais velhos. Paralelamente ocorre um aumento da permeabilidade intestinal e da acumulação de ferro no intestino nestes animais, o que apoia a hipótese de que este órgão possui inúmeras características, em comum com o cérebro, que caracterizam a patologia da DP.

A análise da expressão génica de genes reguladores do metabolismo do ferro, tais como *Ferritin heavy chain* (FtH), *Ferritin light chain* (FtL), *divalent metal transporter 1* (DMT-1), *Transferrin receptor protein 1* (TfR-1) e *Ferroportin* (FPN), demonstraram um aumento padrão em animais idosos (52-60

semanas de idade). Como a maior parte do ferro está contido na molécula de protoporfirina do grupo *heme*, a expressão da enzima degradadora do *heme, hemo oxigenase 1* (HO-1), também foi avaliada, observando-se o mesmo padrão. Esta desregulação permite-nos concluir que a acumulação de ferro influencia a homeostase do ferro e o seu armazenamento no intestino. Os resultados *in vivos* indicam que o stress oxidativo, é presumivelmente impulsionado por um aumento da acumulação de ferro no intestino. Assim, estes resultados validam a hipótese de que a acumulação de ferro pode aumentar a inflamação e que esta elevada concentração de ferro pode impulsionar o stress oxidativo.

A CP é uma proteína produzida em resposta a estímulos inflamatórios, e a pesar da sua função como quelante de ferro ainda não estar estabelecida é uma ideia proeminente. A expressão desta proteína é mais elevada em ratinhos idosos, realçando o fato de que o aumento da idade amplifica a inflamação. Para reforçar estes resultados, injetamos intraperitonealmente CP previamente purificada e observamos uma diminuição nos níveis de ferro no intestino em animais velhos que foram injetados, o que demonstra que esta proteína possui potencial eliminador de ferro. A associação entre a CP e o ferro foi então demonstrada através da avaliação da expressão desta proteína em ratinhos tratados com *heme*. Tendo em conta que o heme possui um efeito pré-inflamatório, mimetiza, de certa forma, o que acontece durante o envelhecimento. Os níveis de CP são superiores em ratinhos novos injetados com *heme*, em comparação com os mais velhos, uma vez que atua como eliminador do ferro, e as injeções repetitivas de *heme* provavelmente saturaram a sua capacidade de ligação. Estes resultados são importantes porque demonstram uma associação entre o aumento da acumulação de ferro no intestino e a maior suscetibilidade a doenças neurodegenerativas, como a DP.

O MPTP é uma neurotoxina que destrói os neurônios dopaminérgicos da *substantia nigra* do cérebro, resultando em sintomas permanentes, característicos da DP. Esta é uma estratégia que tem sido usada para estudar os modelos desta doença em vários estudos com animais. Os animais mais velhos, possuem uma certa inflamação crónica, que lhes é oferecida devido ao avanço da idade. Assim, possuem níveis de CP reduzidos em comparação com os ratinhos novos, o que se justifica pelo fato de estarem sujeitos a um aumento mais reduzido nos níveis de inflamação.

A Deferiprona é um fármaco quelante de ferro, usado para diminuir a acumulação de ferro no intestino e é muitas vezes usado como estratégia terapêutica para prevenir a morte celular programada mediada por ferro, também conhecida como ferroptose. A relevância do ferro nesta doença neurodegenerativa é demonstrada pelos resultados bem-sucedidos obtidos em pacientes com DP em terapia como quelantes de ferro, como a deferiprona, que está agora em estudo clínico de Fase II na maioria dos países do mundo. De facto, a exposição de ratinhos, conhecidos por apresentar maior risco de desenvolvimento de DP, como os mais idosos, após tratamento com deferiprona, leva a uma diminuição significativa da acumulação de ferro. O que nos permitiu concluir que, a inflamação intestinal diminui com o tratamento, como atestado pelos baixos níveis de CP determinados.

Como já foi referido, a acumulação de α-syn é a principal responsável pelo desenvolvimento da DP. Assim, a injeção de α-syn previamente purificada em animais jovens permitiu-nos demonstrar um padrão inflamatório causado pela presença desta proteína. No entanto, será necessária a realização de mais experiências para compreender os níveis diminuídos de CP na presença de heme.

A administração exógena de CP em animais mais velhos, conduziu a uma diminuição nos níveis de ferro no intestino em comparação com os animais não injetados. A administração de CP em ratinhos velhos levou a uma diminuição da rutura epitelial intestinal, que como se sabe é um fator crucial para distúrbios inflamatórios. Estes resultados, permitam considerar a CP como um potencial biomarcador de inflamação intestinal e como ferramenta prognóstica do início da DP causada pela inflamação intestinal.

Em suma, os nossos resultados realçam a hipótese da existência de uma sinalização que ocorre entre o intestino e o sistema nervoso central. O envelhecimento é um dos principais riscos para o desenvolvimento de doenças neurodegenerativas e um dos fatores impulsionadores de inflamação intestinal, através da acumulação de ferro e aumento da permeabilidade intestinal, no cérebro e intestino.

Os ossos resultados mostram que a inflamação está correlacionada com o aumento da acumulação de ferro no intestino e pela presença de α-syn.. Os níveis de CP permitem-nos prever a presença de inflamação intestinal e a gravidade da DP, correlacionando-se com a presença de agregados α-syn no intestino. Finalmente, a restauração dos níveis adequados de CP pode reduzir a incidência de DP e / ou retardar a progressão desta, já que atua como eliminador de ferro. Estes são resultados promissores que apoiam a capacidade da CP em atuar como biomarcador de inflamação intestinal induzida pelo ferro no desenvolvimento da DP.

Estudos mais aprofundados acerca da interação entre a CP, ferro e α-syn serão realizados no futuro, com vista a explicar os baixos níveis de CP na presença de heme e α-syn. Este trabalho permitiu-nos concluir que a CP tem relevância como mediador de inflamação provocada por ferro e como como método preventivo do desenvolvimento da DP, ideias que adicionam conhecimentos a um campo ainda pouco desenvolvido.

Palavras-chave: Calprotectina, inflamação, ferro, envelhecimento e alfa-sinucleína.

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List of Acronyms and Abbreviations

α-syn	Alpha-synuclein
AD	Alzheimer's Disease
DCF	CM-H_2DCFDA
CP	Calprotectin
DLB	Dementia with Lewy Bodies
DMT-1	Divalent metal transporter 1
EEC	Enteroendocrine cells
ELISA	Enzyme-Linked Immunosorbent Assay
FC	Faecal Calprotectin
FPN	Ferroportin
FtH	Ferritin heavy chain
FtL	Ferritin light chain
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
HO-1	Heme oxygenase
IBD	Inflammatory Bowel Disease
IL-10	Interleukin 10
IL-6	Interleukin 6
LB	Lewy bodies
MOM	Mitochondrial Outer Membranes
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MW	Molecular Weight
NAC	Non-Amyloid Component
PD	Parkinson Disease
qRT-PCR	Reverse transcription polymerase chain reaction quantitative real time
ROS	Reactive oxygen species
Rpm	Rotations per minute
SDS-PAGE	Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TfR-1	Transferrin receptor protein 1
β-syn	β-synuclein

1. Introduction

1.1. Parkinson's Disease

Parkinson's disease (PD) is a common age-associated neurodegenerative disease (Melki, 2018). It is the second-most common neurodegenerative disease after Alzheimer's (AD), with a prevalence that increases significantly with advancing age (Dorsey and Bloem, 2018).

PD is a locomotor disorder with symptoms that manifest slowly and progressively over time. The main PD manifestations are tremor, akinesia or bradykinesia, muscle rigidity, and impaired balance and posture. However, loss of smell, sleep disorders, and gastrointestinal symptoms are also common (Liddle, 2018).

PD is characterized by the progressive loss of dopaminergic neurons (DNs) in the s*ubstantia nigra* of the brain, to which the formation of Lewy bodies, composed mostly by alpha-synuclein aggregates, mostly contributes. Although the etiology of PD is not fully elucidated, it is known that protein misfolding and aggregation play a critical role, given the importance of alpha-synuclein in regulating brain functionality and structure. This led the aggregation of misfolded alpha-synuclein to become the hallmark of neurodegenerative diseases, such as PD, Dementia with Lewy Bodies (DLB) and neurodegeneration with brain iron accumulation type 1 (NBIA-1) (Davie, 2008). Because of this feature, these pathologies are collectively called synucleinopathies, and share the important characteristic that once the first aggregates are formed, this process is very difficult to stop or reverse (Linse *et al.*, 2014).

1.2. Alpha-synuclein

In 1988, Maroteaux and his colleagues identified the first member of the synuclein family (George, 2001). Later, the alpha-synuclein (α-syn) gene was mapped and identified on chromosomes 4q21.3-q22 of the human genome. The other member of this family is β-synuclein (β-syn), which is present on chromosome 5q35 (Spillantini, Divane and Goedert, 1995). They share a characteristic consensus sequence KTKEGV that is repeated about six times at the N-terminal part of each protein (Jakes, Spillantini and Goedert, 1994). Besides their high level of homology, α- and β-syn differ in non-amyloid component (NAC) regions. β-syn has 11 amino acids less (amino acids 73–83), which result in profound consequences for its aggregation (Goedert and Spillantini, 2012). Once these proteins precipitate, they are located near synaptic vesicles, as described by Clayton and George in 1999, according to whom α- and β-syn form a moderately dense, amorphous "matrix" just behind or beneath this region (Clayton and George, 1999).

α-syn is formed by 140 amino acids. It contains a N-terminal domain (residues 1-60), a central hydrophobic portion (residues 61-95), and a C-terminal region (residues 96-140). The amphipathic N-terminal region contains four 11-residue repeats, which includes a consensus sequence (KTKEGV). This domain is important for the normal function of this protein and mutations in this segment have been associated with PD development (Davidson et al., 1998). The central hydrophobic part of α-syn includes the NAC region, which has been described as involved in protein aggregation. Indeed, deletion of large segments within this motif is likely to diminish α-syn oligomerization fibrillogenesis in vitro (Giasson et al., 2001)(Giráldez-Pérez et al., 2014). The C-terminal is a highly acidic and proline-rich region that does not seem to play a role in α-syn fibrillation but it rather serves to maintain the natively unfolded structure of the protein (Qin et al., 2007).

α-syn is located predominantly in terminal axons of mature neurons, including olfactory neurons and basal cells. However, this protein was also detected in hematopoietic cells, platelets, and cardiac tissue,

suggesting that α-syn does serve only for synaptic development, as also indicated by experiments conducted with animals genetically deprived of α-syn (Norris, Giasson and Lee, 2004).

Nevertheless, most of the literature on synuclein proteins refers to a major role played in nervous system development and synaptic plasticity maintenance. This includes a major involvement in synaptic modulation or maturation, which justifies synucleins expression only after synaptic development (Murphy *et al.*, 2000). So far, one of the main functions of α-syn is to regulate the release of neurotransmitters in synaptic vesicles. However, it has been described as also capable to modulate the levels of glucose, to exert chaperone activities, to promote neuronal differentiation and to modulate dopamine biosynthesis (Emamzadeh, 2016).

A number of evidences indicate an important role of α-syn on preventing mitochondrial function in the pathogenesis of neurodegenerative diseases such as PD and AD. In 2010, Kamp and his colleagues conducted in vitro studies showing that α-syn was capable to inhibit the membrane fusion of this organelle. This suggested that binding of α-syn to the outer mitochondrial membrane might modify its curvature (Kamp et al., 2010) and subsequent morphology with a mechanism that is still not fully understood.

It has also been reported that α-syn can promote amyloid β-fibrillogenesis, thus being relevant also in AD (Kaplan, Ratner and Haas, 2003). However, although it remains unclear how these two proteins link themselves, β-amyloid (Aβ) and α-syn can interact directly under pathological conditions and lead to the formation of toxic oligomers that result in oxidative stress (Crews et al., 2009)(Twohig and Nielsen, 2019).

1.3. Pathology

PD patients have low levels of dopamine in the substantia nigra of the brain, caused by a progressive loss of dopaminergic neurons (DNs). The substantia nigra is part of the basal ganglia, which includes a group of structures regulating body movement. During PD the interruption of "movement" information to the striatum and motor cortex leads patients to develop locomotor dysfunctions.

One of the features that contributes to DNs degeneration is the accumulation of α-syn aggregates. α-syn first form oligomers, then fibrils, and finally Lewy bodies (**Figure 1.1**). The nucleation process begins with an initial lag phase in which a-syn monomers are in their native and unfolded form. Then, they create structures that aggregate and finally form ordered oligomeric nuclei. A conformational change exposes the NAC domain, which may interfere with hydrophobic interactions and initiate the aggregation process. During the growth phase, the nucleus adds monomers to form, first, what are called larger oligomers, then protofibrils, and finally fibrils. In the last phase, known as stationary, fibrils, and monomers appear to be in equilibrium (Deleersnijder et al., 2013) (Giráldez-Pérez et al., 2014).

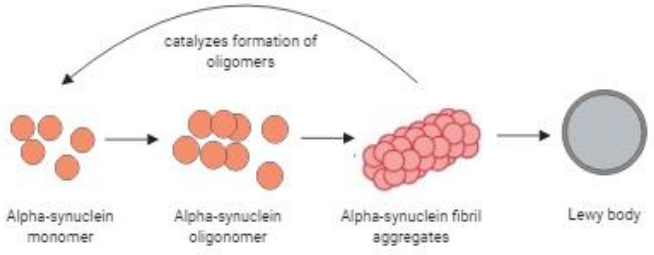

Figure 1.1 The accumulation of α-syn aggregates leads to the formation of first oligomers, then fibrils, and finally Lewy bodies.

The pathological aggregation of α-syn could be enhanced by the use of pesticide, such ,as rotenone and β-Hexachlorocyclohexane as well as by the presence of genetic mutations in the SNCA gene, among causes that are still unknown (Betarbet *et al.*, 2006)(Richardson *et al.*, 2011)(Deleersnijder et al., 2013). Apparently, it seems that α-syn binds to membranes as an extended α-helix or a broken-helix, through a mechanism poorly understood. Yet, oligomers are known to cause membrane permeabilization and subsequently disruption of cell homeostasis (Stöckl, Zijlstra and Subramaniam, 2013)(Giráldez-Pérez et al., 2014). The interaction of α-syn with other proteins, such as FK506 binding proteins, which presence accelerate fibril formation, have been studied but are not yet completely clear (Deleersnijder et al., 2013).

Misfolded α-syn (10-30%) fibrils form eosinophil cytoplasmic inclusions, which are known as Lewy bodies (LB), albeit recent studies have suggested that Lewy pathology in Parkinson's disease consists only of crowded organelles and lipid membranes (**Figure 1.2**) (Bonini and Giasson, 2005)(Schulz-Schaeffer, 2010)(Shahmoradian *et al.*, 2019). The appearance of these aggregates precedes motor symptoms and correlates with advanced stages of the disease. During the last decade, it has been proposed that the propagation of LB is mediated by a cell-to-cell transmission of misfolded α-syn onto recipient cells (Desplats et al., 2009).

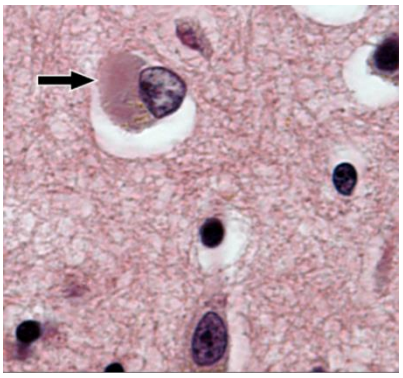

Figure 1.2 Lewy Body in the motor cortex. (Henderson V. W., 2017) "Dementia with Lewy Bodies" Stanford ADRC

Interestingly, LB have been found in the enteric and peripheral nervous system of post-mortem samples from PD patients. This discovery raised the hypothesis that PD can start developing in the gut and affect the brain just afterwards. This postulate is known as "the Braak hypothesis" and suggests that the formation of α-syn aggregates may spread from the gut to the brain through the vagal nerve. Once accumulating in the brain, these aggregates would then be responsible for DNs death (Braak et al., 2003). As PD progresses, the severity of clinical manifestations increases. Six stages characterize PD development. According to the evolution of the disease, the affected neurological structure and the symptoms that patients suffer from might vary. These are then classified into: non-motor symptoms observed during stage 1 and 2; motor symptoms during stage 3 and 4; and cognitive symptoms during stage 5 and 6 (Braak et al., 2003)(Visanji et al., 2014).

The Braak hypothesis was further supported in 2019, when Kim and his colleagues showed that the intraperitoneal injection of α-syn into in mice leads to the formation of pathological forms of this protein, which are then capable to migrate into the brain through the vagal nerve (Kim et al., 2019).

1.4. Iron accumulation

One of the main catalysts for α-syn aggregation is iron. Iron is as an essential element, necessary to keep cellular homeostasis. It is required for all physiological processes, including DNA replication and

survival (Lingor, Carboni and Koch, 2017). The amount of iron in the body is tightly regulated and unbalances in iron metabolism have been linked to a variety of pathological conditions, including neurodegenerative diseases like PD. Iron is absorbed in the intestine and release into bloodstream (Morgan and Oates, 2002), a process that occurs in three stages: uptake of iron by enterocytes; intracellular trafficking and extracellular transport into circulation (Wessling-Resnick and Chung, 2003). The concentration of iron in the intestine gradually increases in early adulthood until 40 years of age, above that it remains constant. At this point, an increase in iron level has been shown in the basal nuclei of enterocytes, which are linked to the neural network of the gut that has been involved in PD (He et al., 2015).

An increased expression of the intracellular iron storage ferritin has been detected in the gut with advancing age, which further confirms that iron accumulates in this compartment as we get older. The main regulator of iron homeostasis is hepcidin, a peptide hormone produced by hepatocytes (Ganz, 2001). It binds to ferroportin, which is the only known iron exporter so far, and regulates the absorption of iron in the duodenum (Ganz and Nemeth, 2011). Thus, when iron is needed, hepcidin is downregulated. However, pro-inflammatory cytokines induce both the production and secretion of hepcidin, as confirmed by its elevated levels in patients with chronic inflammation (Nemeth et al., 2003)(Nemeth et al., 2004). Aging is a chronic and sub-acute inflammatory state, which increases hepcidin and decreases ferroportin expression (Ferrucci et al., 2010). This indicates that elderly might suffer from low iron into circulation while presenting tissue iron accumulation. Whether gut iron retention favors the aggregation of α-syn in this organ could be the case and object of our studies.

1.4.1. Iron and Parkinson's Disease

In addition to α-syn accumulation, brain iron overload was postulated as one of the leading causes of neuronal death. Importantly, iron chelation therapy has been used against this pathology and the use of deferiprone is nowadays in Phase II clinical trial in most countries worldwide.

The brain is the organ presenting the highest concentration of iron, after the liver. Iron is mostly abundant in the basal ganglia and the disruption of its homeostasis justifies the occurrence of pathologies starting in this area, like PD (He et al., 2015) (Lingor, Carboni and Koch, 2017). Unbalanced iron levels occur with advancing age, further demonstrating that aging is the major risk factor for the development of this neurodegenerative disease. Also ferritin expression is higher in elderly and mainly detected in cortex and hippocampus, besides the basal ganglia (Connor et al., 1992). One of the largest reservoirs of iron in dopaminergic neurons is a protein known as neuromelanin, which role is to chelate this metal and prevent its participation into the Fenton chemistry to form highly reactive hydroxyl radicals. Its neuroprotective action, however, decays with PD progression and a decrease in neuromelanin expression is observed in later stages of PD when compared to early phases. The degeneration of DNs releases neuromelanin-iron complexes in the extracellular environment, which are engulfed by microglia. Whether this contributes to activate neuroinflammation and promotes neurodegeneration seems to be the case (Gerlach et al., 2003)(Zhang et al., 2011).

The reactivity of iron relies on its ability to exchange electrons among different substrates. Its unbalance leads to oxidative stress and the formation of radical oxygen species is one of the etiological causes of PD. Since iron acts as cofactor for tyrosine hydroxylase, the enzyme responsible for dopamine synthesis, the disruption of iron homeostasis has a direct impacts on dopamine levels (Beard, Erikson and Jones, 2013) (Deng et al., 2017). Moreover, taking into account that dopamine catabolism already triggers oxidative stress, the slight impairment in iron metabolism turns the substantia nigra susceptible to iron-driven neuronal death (Zecca et al., 2004)(Galvin, 2006). Since both iron and oxidative stress have been

shown to favor the aggregation of α-syn, one can understand how Lewy bodies have been associated to the pathogenesis of PD (Li et al., 2011).

Although the absence of a direct correlation with α-syn, recent studies found that patients with lower hemoglobin levels were more susceptible to PD. Hence, this points to anemia as a risk factor for this pathology, as demonstrated by the reduced levels of ferritin and iron in the circulation of PD patients (Deng et al., 2017). Interestingly, population-based studies refer to anemia as a risk factor only if PD is developed by men. The same values of blood parameters in women were not associated with an increased incidence of PD (Rozani et al., 2019).

1.5. Calprotectin

Anemia is often associated with tissue iron accumulation and the gut is not an exception. Increased iron levels in the intestine were linked to enhanced inflammation and result in higher calprotectin (CP) expression. CP belongs to S100 proteins family and has been involved in the pathogenesis of PD. It regulates many cellular functions, such as proliferation, differentiation, apoptosis, calcium homeostasis, energy metabolism, etc (Donato *et al*., 2012).

Human CP exists either as a heterodimeric (αβ) or heterotetrameric (α2β2) form of S100A8 (α) and S100A9 (β), which are also known as MRP8 and MRP14, respectively. They represent the largest subfamily of the EF-hand calcium-binding proteins and are capable of sequestering transition metals like iron, manganese, and zinc via a chelation mechanism that occurs in the presence of calcium (Nakashige et al., 2015) (Vogl, Gharibyan and Morozova-Roche, 2012) (Clark et al., 2015).

S100A8/A9 is expressed by immune cells, such as neutrophils and monocytes (Stříž and Trebichavský, 2004)(Ehrchen et al., 2009), from which it is released along with pro-inflammatory cytokines, ROS and nitric oxide (Benoit, Desnues and Mege, 2008). In addition to immune-mediated gut dysfunction, S100A8/A9 is highly produced during trauma, infection, stress and many other inflammatory processes (Ometto et al., 2017). CP interacts with cytoskeleton proteins, including tubulin, microfilaments, and keratin intermediate filaments (Vogl et al., 2004). It is involved in cytosol tubulin polymerization and cytoskeleton modulation, these being important for granulocyte migration and elucidating the role of CP in recruiting neutrophils during inflammation (Wang et al., 2018).

The ability of CP to bind iron allows hypothesizing that CP might act as iron scavenger. This notion is supported by the observation that this protein binds only the reduced and most reactive form of iron, i.e. Fe(II) (Nakashige et al., 2015). The anaerobic environment of the gut impedes the oxidation of Fe(II) to Fe(III), thus turning it a perfect compartment for CP to work. It was recently reported that CP can bind Fe(II) and block microbial Fe(II) uptake, thus depriving bacteria of this essential nutrient (Nakashige et al., 2015)(Nakashige and Nolan, 2017). This supports the idea that CP could influence iron redox speciation by playing a physiological role in iron homeostasis at the host-pathogen biological interface (Nakashige and Nolan, 2017). Since Fe(II) also favors the aggregation of α-syn, the neutralization of iron reactivity by CP, might prevent and/or delay PD development.

CP is reported as a potential diagnostic marker of inflammation. (Aadland *et al.*, 2002)(Jonsson *et al.*, 2017). Thus, while decreasing CP expression could represent a potential therapeutic approach against gut inflammation, its increase might prevent iron accumulation to foster gut-driven PD development (Benet Bosco Dhas, Vishnu Bhat and Bahubali Gane, 2012).

1.6. Calprotectin and inflammation

The involvement of the gut-brain axis in PD and resulting inflammatory activation (Tansey and Goldberg, 2010) point at the measurement of CP as a potential prognostic and diagnostic tool. The severity of PD might correlate to the levels of CP produced in the gut and released into circulation.

Indeed, inflammation can trigger the production of S100A9, which then promotes its transcription via positive feedback mechanisms activating the NF-kB signaling pathway. Whether different ratio of S100A8 and S100A9 expression could favor neurodegenerative diseases is a possibility that needs to be further explored (Swindell et al., 2013). However, the use of CP as potential biomarker, capable to predict disease severity, is supported by previous observations correlating increased levels of this protein to severe cardiovascular, autoimmune and bowel diseases (Xia et al., 2018). In the latter, quantification of CP in blood and feces was shown to predict the extent of gut inflammation, thus aiding physicians to prescribe the correct therapeutic treatment to their patients (Sherwood and Walsham, 2016)(Freeman et al., 2019). Whether individuals diagnosed with bowel inflammation are at higher risk of PD development remains to be fully established (Villumsen et al., 2019)(Weimers et al., 2019). However, gastrointestinal problems and constipation are frequent complains by more than 80% of PD patients, which manifest over a decade before the development of motor symptoms, indicating that the gut plays a crucial role in PD development.

The figure below is an attempt to describe the link between α-syn, CP and iron and their correlation with PD onset. The accumulation of iron in the gut with advancing age activates the immune response, which results in the release of CP into circulation. By seeking to scavenge as much iron as possible, increased levels of CP might also prevent the formation of α-syn aggregates, whose production is enhanced by iron (Kehl-Fie et al., 2011).

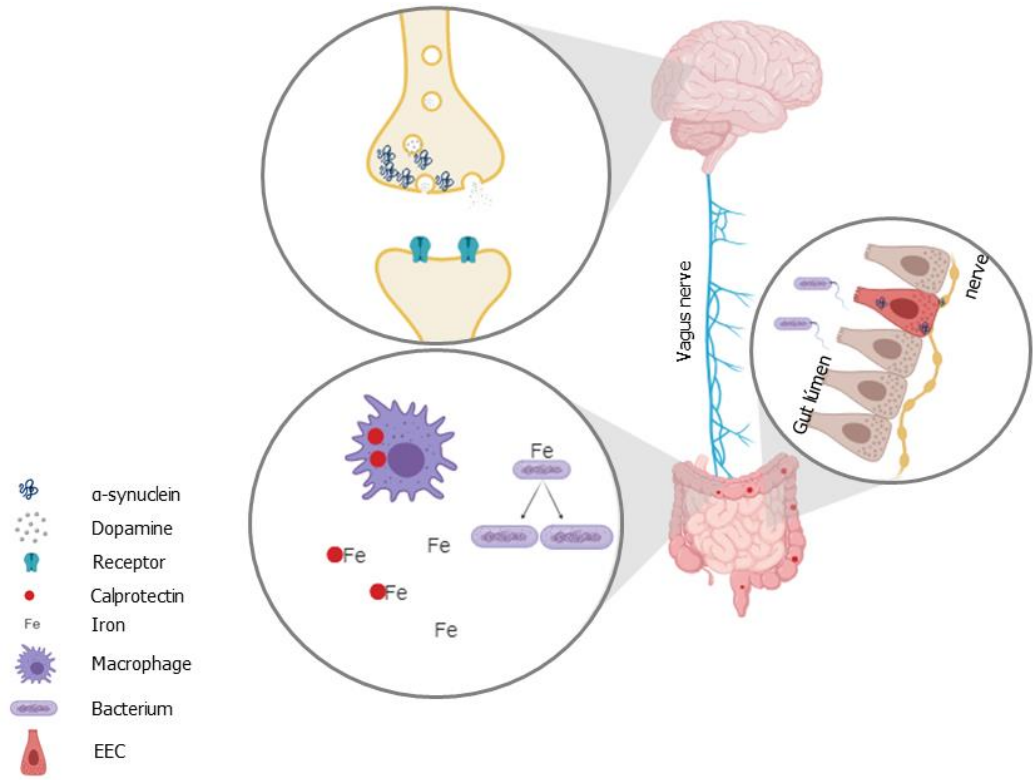

Figure 1.3 (A) Intestinal inflammation leads to the release of S100 proteins, including CP (S100A8/A9). Its high affinity for iron (II) allows CP to act as iron scavenger. (B) Increased iron levels favor the aggregation of α-syn, which once formed in the gut is possibly transported to the brain, through the vagal nerve and efferent neurons. (C) In the central nervous system, the accumulation of α-syn contributes to DNs loss and the development of PD.

2. Objectives

There is increasing evidence suggesting a direct correlation, mediated by the vague nerve, between the brain and the intestine. The gut is associated with several inflammatory processes of its own cells with an established relationship with iron. Increasing concentration of this ion, leads to inflammatory conditions. Calprotectin is one of the most important proteins with a role in the development of chronic inflammation, becoming a marker for inflammatory syndromes, can also bind Fe(II) and could be a form of scavenging. Since Iron in its reduced form also favors the aggregation of α-syn, the neutralization of iron reactivity by CP, might prevent and/or delay the development of PD. The aims will be achieved by biochemical and cellular approaches combined with studies in mice as animal model. The findings obtained will allow establishing the levels of calprotectin as biomarkers for PD severity and alpha-syn aggregation.

Hence, the aims of this thesis were:

1. Assess whether low-grade inflammation correlates to an increased iron accumulation in the gut and the emergence of protein aggregation, such as a-syn, in this compartment.
2. Assess whether low-grade inflammation and iron accumulation in the gut correlate to the expression of calprotectin in this compartment and into circulation.
3. Assess whether the level of calprotectin can predict the severity of PD and correlate to the formation of alpha-synucleins aggregates in the gut.
4. Assess whether the re-establishment of proper levels of calprotectin can reduce PD incidence and/or delay PD progression.

3. Materials and Methods

3.1 Cells and culture conditions

Human intestinal Caco-2 cells (ATCC 37-HTB), derived from human colon adenocarcinoma, were grown in Minimum Essential Medium (GIBCO), supplemented with 15% fetal bovine serum, 25 mM HEPES, 100 units ml−1 penicillin and 100 μg ml^{-1} streptomycin. Culture medium was changed every 2 to 3 days and cells were split every 7 days, upon enzymatic removal with 0.25% trypsin-EDTA, 5 min at 37 °C. Caco-2 cells were kept in a humidified atmosphere of 5% CO2–95% air and treated with heme and/or iron, as indicated in Results. Viability assays were conducted upon Heme or Iron in 96-well plate (15,000 cells / well) for 24 hours, while ROS measurement were performed after 4 hours treatment in a 12-well plate (150,000 cells / well), using concentration of Heme or Iron in agreement with previous data.

3.2 ROS Measurement

Oxidative stress was measured by flow cytometry, using cell-permeable probes capable to quantify total ROS, as with 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein di-acetate, acetyl ester (CM-H_2DCFDA) (Life Technologies, Ref. No. C6827), and mitochondrial ROS when using MitoSOX™ (Thermo Fisher, Ref. No. M36008). Briefly, Caco-2 cells were cultured in 12-well plates for 48 h and treated with iron or heme for 4 h. Once detached, cells were transferred to a 2 mL tube, centrifuged at 1000 rpm for 5 minutes, and washed with 500μL of PBS 1X. Cells were then resuspended in 200μL of PBS 1X, to which 1μM CM-H_2DCFDA (DCF) or 1μM MitoSOX™ was added. Cells were incubated for 15 minutes at 37ºC, being protected from light. Subsequently, cells were washed, resuspended in 100μL of PBS 1X, and analyzed by Flow Cytometer (BD FACSCanto II - BD Bioscience).

3.3 Mice

C57BL/6 mice were used as in vivo animal model. All animals were kept under specific pathogen-free conditions, according to the Animal Care Committee of the Champalimaud Foundation. Comparison between 8-12-week-old (young) and 52-60-week-old (old) animals were carried as explained in Results.

3.4 ELISA

Proinflammatory cytokines, like interleukin-6 (IL-6), or calprotectin were measured by ELISA, using Mouse IL-6 (Invitrogen, Ref. No. 88-7064-22) and calprotectin ELISA kit (Elabscience, Catalog No. E-EL-M1143), respectively, according to manufacturer's instruction.

3.6. Disruption of gut epithelium

C57BL/6 mice were injected intravenously with 100 L of 2% Evans Blue solution. The animals were then sacrificed and perfused with 20 mL of saline buffer. The gut was harvested, flushed to remove fecal content and left 48h in formamide. The extent of gut disruption was then measured by quantifying the extravasation of the Evans Blue dye into the intestine. Absorbance was read at λ=620nm in a spectrophotometer (Biotrak II Plate Reader, Amersham Biosciences).

3.7. Induction of Parkinson's disease

C57BL/6 mice were injected intraperitoneally with 200μl of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; 15mg/kg, 3 injections every 2 h) or of purified α-syn (x mg/kg, 5 injections

every 24h). The animals were monitored daily and locomotor dysfunction was assessed as time of performance, i.e. the seconds that mice placed up-side-down on a vertical pole need to descend it.

3.8. Iron measurement

Gut samples were dried (24h, 99 ºC), dissolved (3M HCl, 10% trichloracetic acid; TCA; overnight at 65 ºC) and diluted (10µl) in water (590µl). β-mercaptoethanol (10µl), sodium acetate (pH 4.5; 500µl) and bathophenanthroline-disulfonic acid (80µl) were added to each sample (37 ºC, 1h). Absorbance was measured at λ=535nm in a spectrophotometer (Biotrak II Plate Reader, Amersham Biosciences).

3.9. RNA isolation, cDNA synthesis, and quantitative real time-PCR

Once harvested the gut, RNA extraction was performed using the NucleoSpin® Blood kit (Macherey-Nagel, Ref. No. 740951.50) according to manufacturer's instructions. NanoDrop 2000c spectrophotometer (Thermo Scientific) was used to determine the quantity and quality of the RNA. Total RNA was reverse transcribed into cDNA using a SuperScript III First-Strand Synthesis System for quantitative real-time PCR (RT-qPCR) (Invitrogen, Ref. No. 18080051), according to manufacturer's instruction. RT-qPCR was performed using the primers indicated in the Table below and samples were run on an ABI QuantStudio-384 (Applied Biosystems). *GADPH* was used as housekeeping gene and samples were run in duplicates.

1Table 1. List of primers used in this study for RT-qPCR analysis. The expression of genes involved in iron/heme metabolism was assessed by qRT-PCR.

Function	Gene	Primer forward (5'-3')	Primer reverse (5'-3')	Annealing Temperature
Iron metabolism	TfR-1	TGTGACCTGTGTATTGGCCC	GCAGGGTTCTTTCCTTCGGT	44ºC
	DMT-1	GCAGTGGTTAGCGTGGCTTATT	AGACAGACCCAATGCAATCAAA	41ºC
	FtH	CCATCAACCGCCAGATCAAC	GCCACATCATCTCGGTCAAA	42ºC
	FPN	AGGTGACACTATAGAATATGCCTTAGTTGTCCTTTGGG	GTACGACTCACTATAGGGAGTGGAGAGAGAGTGGCCAAG	60ºC
Heme catabolism	HO-1	TGACACCTGAGGTCAAGCAC	TCTCTGCAGGGGCAGTATC	44ºC
Calprotectin and α-syn	S100A8	TGTCCTCAGTTTGTGCAGAATATAAA	TCACCATCGCAAGGAACTCC	44ºC
	S100A9	GGTGGAAGCACAGTTGGCA	GTGTCCAGGTCCTCCATGATG	44ºC
	α-syn	AACTGCAGTTCTTCAGAAGCCTAGGGAGC	GCTCTAGACTGGGCACATTGGAACTGAG	47ºC

3.10.Statistical analysis

Data was expressed as mean values □ standard deviation. Statistically significant differences between indicated conditions were assessed using One-way non-parametric ANOVA (Kruskal-Wallis test). When significant, this was indicated as *p < 0.05; ** p < 0.01; ***p < 0.001. All statistical analysis was performed using GraphPad Prism (v. 5.0) (GraphPad Software).

3.11.Alpha-synuclein Expression and Purification

Alpha-synuclein was expressed in *E.coli* BL21 (DE3) competent cells, which were transformed for high-efficiency of protein expression. 2% of plasmid (Pgs-21A- Miniprep 24/01/2013) was added to competent cells, kept on ice for 30 minutes and then heated for 54 seconds at 42ºC. These were then chilled on ice for 5 minutes and grown in 960 µl of Lysogeny Broth (LB) at 37ºC (250 rotations per minute (rpm) for 1 hour). 100 µL of cell suspension was then mixed with 100 µg/mL ampicillin, spread in a LBA plate and grown overnight at 37ºC. One colony was then isolated, added to 50 mL LB with 100 µl/mL Ampicillin and grown overnight at 37ºC (150rpm). Then, 20% of cell suspension was transferred to 0.5 L fresh LB with 100 µl/mL Ampicillin and grow at 37ºC until OD_{600} = 0.5-0.6. At this point, α-syn, expression was induced by adding 0.5 mM of IPTG. After 4h incubation, cells were collected by centrifugation (JA-14 rotor) at 8,000 rpm (10 min at 4°C).

Purification of α-syn starts by resuspending cells in 40mM TRIS pH=8.3, 1mM EDTA (100mL buffer to 15g cells), with DNase and 1mM PMFS protease inhibitor. After solubilization, cells were sonicated (50% amplitude and 0.5% on/off cycles, for 15minutes), centrifuged (JA-20 rotor) at 20,000rpm for 20 minutes, heated at 80 °C for 20 minutes and then centrifuged again at the same speed and conditions. Supernatant was stirred for 30 minutes at 4 °C and centrifuged again at 13,500rpm for 20 minutes. Supernatant was then collected, filtered and loaded into an ionic-exchange chromatography column (HiPrep 16/10 Q-Sepharose fast flow equilibrated with 20mM TRIS pH:8.0) with 0 to 1M NaCl ionic gradient.

6.5g of *E. coli* cells expressing recombinant human α-syn were used in the first purification phase. After the three first steeps of purification, the extract of proteins was then submitted through an ionic-exchange chromatography column with an ionic gradient from 0 to 1M NaCl. The result of this step can be observed below (**Figure 3.1**).

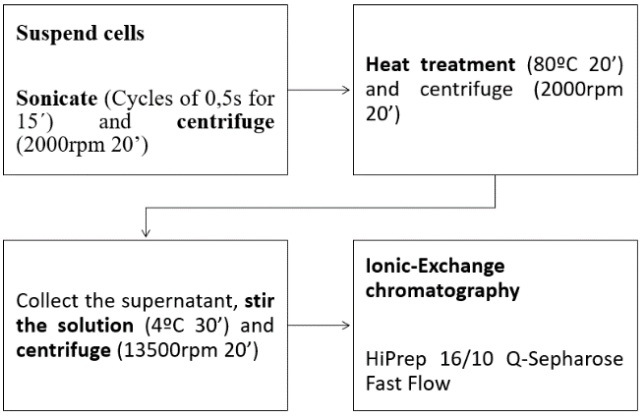

Figure 3.1 Diagram of purification steps for α-syn.

The α-syn was eluted at 19% of buffer B, i.e. 280mM of NaCl, and by analyzing the SDS-PAGE gel in **Figure 3.2**. The protein was then frozen at -80ºC.

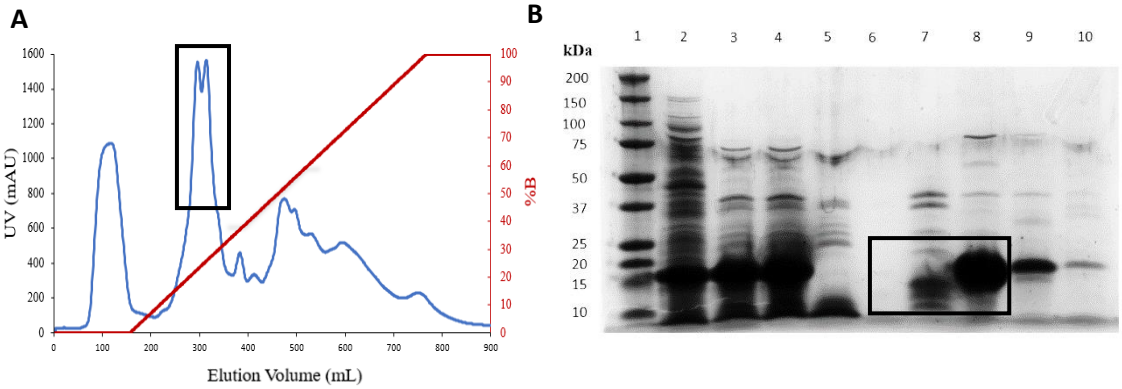

Figure 3.2 Ionic-exchange chromatography purification step. (A) Chromatogram with Abs280nm (black line) and buffer B% (red line) vs elution volume (Ve). (B) SDS-PAGE showing purified aSYN: 1- marker; 2- product of 1ºcentrifugtion; 3- product of 2ºcentrifugation; 4- fraction injected; 5- unbound; 6,7- fractions from 1st peak; 8- fraction from the 2nd peak; 9,10- fraction between 2nd and 3th peak.

3.12. Calprotectin Expression and Purification

Recombinant human S100A8/A9 was expressed in E.Coli BL21 (DE3) competent cells, which were transformed with plasmids containing S100A9 and S100A8 gene, respectively by preparing two pre-inocula. S100A8 and S100A9 glycerol cell culture stock were added to DYT medium supplemented with 100mg/mL ampicillin and 20% glucose, and incubated at 37ºC with 150rpm agitation until OD_{600} = 0.7-1.0. Expression was then induced by adding 0.5mM of IPTG. After 4h incubation, cells were collected by centrifugation (JA-14 rotor) at 8,000rpm for 10 minutes at 4ºC, and then frozen.

Purification starts with the separate resuspension of S100A8 and S100A9 containing cells in 20mM TRIS-HCL pH=8.0 (50mL buffer to 10g cells), with DNase and 0.5mM of PMFS. Cells were then sonicated (50% amplitude and 0.5 on/off cycles, for 10 minutes) and centrifuged (JA-20 rotor) at 20,000rpm for 20 minutes to isolate inclusion bodies. The pellet was washed in 20Mm TRIS-HCL pH=8.0, 10Mm EDTA, and 0.5% (v/v) Triton X-100, sonicated and centrifuged. The pellet was resuspended in 6M Guanidine-HCL and incubated for 1 hour on ice under gentle agitation, in other to extract proteins from inclusion bodies. After 1 hour, the extract was diluted 6 times with 20Mm TRIS-HCL pH=8.0, 1Mm EDTA, 1Mm EGTA and 0.5Mm DTT (to decrease guanidine-HCL concentration to 1M), and centrifuged (20000rpm for 20 minutes). The supernatant was filtered and loaded into a desalting chromatography column (HiTrap desalting) to separate proteins from guanidine. The fraction obtained, corresponding to the proteins, was loaded into a size-exclusion chromatography column (Superdex 75 16/600) against 20mM TRIS-HCL, 1mM EDTA, 1mM EGTA, 150mM NaCl pH=8.0 and 0.5Mm DTT. The chromatogram was analyzed, to choose key peaks that were then observed in a SDS gel, to discriminate fractions containing S100A8 and S100A9 homodimers. Finally, fractions were passed through an ionic-exchange chromatography column with a 0 to 1M NaCl ionic gradient. The two elution buffers for this final step were: A) 20mM TRIS-HCL Ph=8.0, 1mM EDTA, 1mM EGTA. 0.5mM DTT and B) 20mM TRIS-HCL Ph=8.0, 1mM EDTA, 1mM EGTA. 0.5mM DTT with 1M NaCl.

The first steps are made separately in view of CP being a heterodimer of S100A8/A9, thus cells were grown independently. Afterward, the two-inclusion body is resuspending in one solution and the pellet resuspends with a Guanidinium solution (**Figure 3.3**).

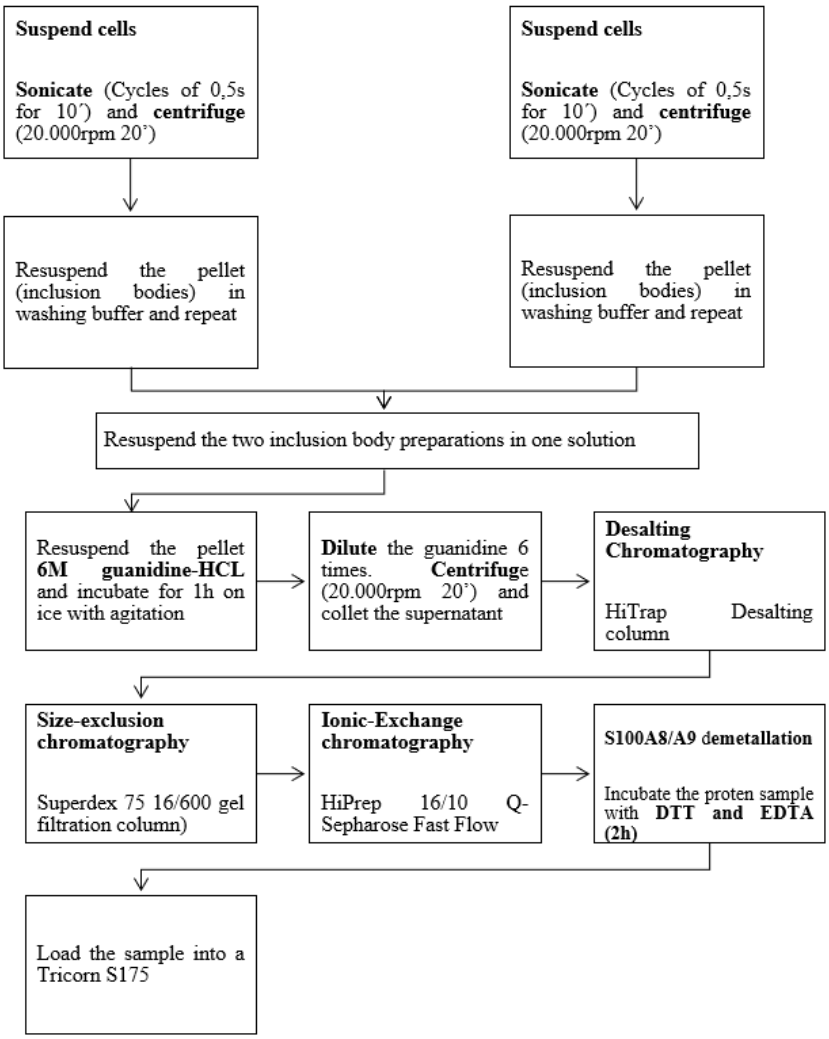

Figure 3.3 Diagram of purification steps for S100A8/A9 heterodimer.

In the first step of purification 17g of E. coli expressing recombinant human S100A9 and 8g of E. coli expressing human S100A8. To isolate inclusion bodies and then extract the proteins within, inclusion S100A8 and S100A, by adding guanidine. The extract was then injected to a desalting chromatography to separate proteins from the guanidine (the prolonged presence of this compound may permanently lead to protein misfolding).

In the chromatogram it is possible to observe the output of the protein fraction followed by the increase of conductivity (red curve) when guanidine passes through the sensor, providing an easy way to discriminate the presence of this compound. It's known that S100A8 and S100A9 has a molecular weight of approximately, 10kDa and 13kDa correspondingly, and the presence of these proteins it is possible to see in **Figure 3.4(B)**.

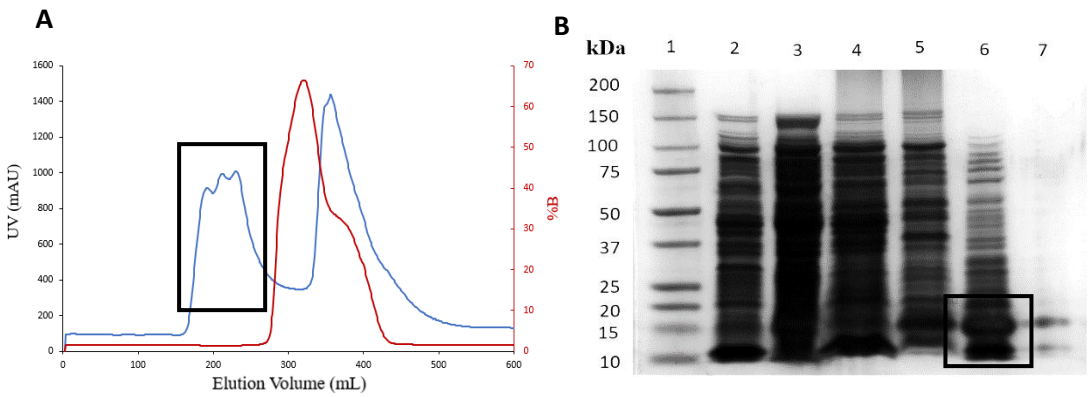

Figure 3.4 Desalting chromatography purification step. (A) Chromatogram with Abs280nm vs elution volume (Ve). (B) SDS-PAGE: 1- marker; 2- supernatant S100A8 (1º centrifugation); 3- supernatant S100A9 (1º centrifugation); 4- supernatant S100A8 (2º centrifugation); 5- supernatant S100A9 (2º centrifugation); 6- fraction from 1st peak; 7- Flow through (result of the concentration of the 1st peak with amicons (3kDa)).

After that, the protein extract was loaded into a size-exclusion chromatography column. In the chromatogram (**Figure 3.5(A)**) are represented the different peaks that contain a variety of proteins. The three different peaks were collected and then charged in the gel. The presence of both, S100A8 and S100A9 are then confirmed by SDS-PAGE (2nd peak).

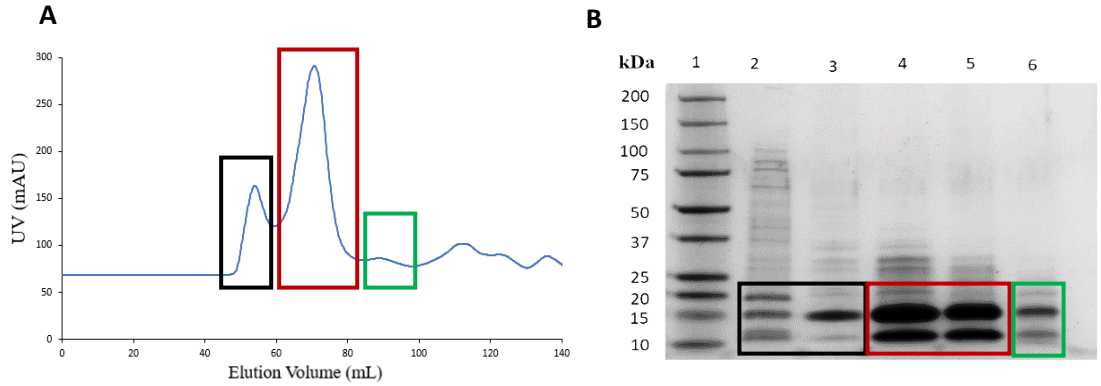

Figure 3.5 Size-exchange chromatography purification step. (A) Chromatogram with Abs280nm vs elution volume (Ve). (B) 1- marker; 2,3- fractions from 1st peak; 4,5- fraction from 2nd peak; 6- fraction from 3th peak.

Last, of all, the fractions collected were then passed through an ionic-exchange chromatography column (HiPrep 16/10 Q-Sepharose Fast Flow) with an ionic gradient from 0 to 1M NaCl. The results are represented in **Figure 3.6**.

It was necessary to demetallize the protein, this way, the sample was incubated with DTT and EDTA and loaded into a size-exclusion chromatography column to obtain purified metal-free S100A8/A9 (apo form).

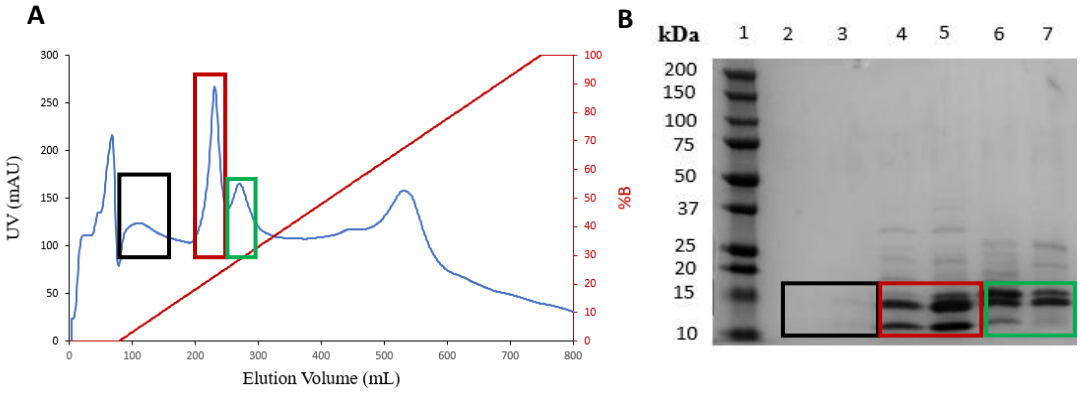

Figure 3.6 Ion-exchange chromatography purification step. (A) Chromatogram with Abs280nm vs elution volume (Ve). (B) 1- marker; 2,3- fractions from 1st peak; 4,5- fraction from 2nd peak; 6,7- fraction from 3th peak.

3.12.1. Calprotectin Demetallation

To remove potential metal ions, bound to S100A8/A9, proteins were incubated 2 hours at 37ºC, in presence of 300-fold protein concentration of dithiothreitol (DTT), to avoid disulfide bond formation, and 0.5mM of ethylenediaminetetraacetic acid (EDTA) to chelate metal ions. After incubation, protein sample (prepared with Chelex water, to avoid the binding of metal ions present in distilled water) was loaded into a size-exclusion chromatography column (Superdex 75) with 50Mm TRIS-HCL pH=8.0 to separate existing multimers, DTT and EDTA, thus obtaining purified S100A8/A9 into the Apo form (demetaled).

4. Results

4.1 Aging triggering inflammation, increased gut permeability and iron accumulation

It is known that aging is the main risk factor for neurodegenerative diseases, like PD. Considered as a sort of sub-acute chronic inflammatory disorder, the presence of inflammatory cells can be observed in both peripheral blood and parenchyma tissues, and their activation might lead to the release of pro-inflammatory cytokines. Hence, an increased level of interleukin 6 (IL-6) was observed in the peripheral blood of 52-60-week-old C57BL/6 mice when compared to younger animals (8-12-week-old), as assessed by ELISA in **Figure 4.1**.

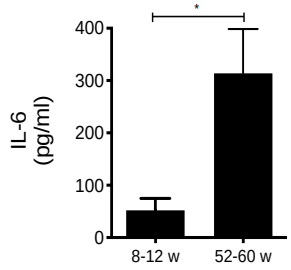

Figure 4.1 Increased level of IL-6 in circulation of mice aged as indicated and assessed by ELISA**.** Results were expressed as mean ± standard deviation (n=10-15 mice per group). Significance has been observed according to t-test.

Increased expression of IL-6 gene was also observed in the gut of old mice mice when compared to young animals. No difference in the expression of the anti-inflammatory cytokine interleukin 10 (IL-10) was detected by qRT-PCR in the intestine of the same mice (**Figure 4.2**).

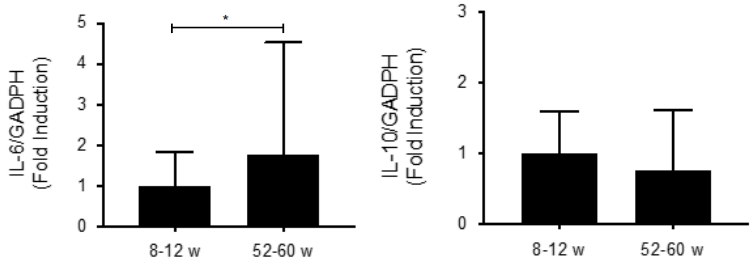

Figure 4.2 Expression of IL-6 and IL-10 in the intestine of mice aged as indicated, measured by qRT-PCR**.** Expression of GADPH was used as loading control. The results were normalized to GADPH and expressed as fold induction in relation to young animals, used as controls, ± standard deviation (n=10-15 mice per group). Significance has been observed only for IL-6 according to t-test.

The gut is known to contribute to the inflammatory state of aging, as disruption of gut epithelial barriers might foster inflammation. When increased gut permeability was assessed by measuring the extravasation of the Evans Blue dye, injected in mice intravenously, disruption of gut epithelium was found in old but not young animals (**Figure 4.3**).

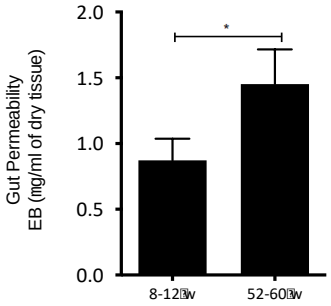

Figure 4.3 Gut permeability was evaluated in the indicated groups of mice. The results were expressed as mean ± standard deviation (n=10-15 mice per group). Significance has been observed according to t-test.

Similar data have been obtained in different projects, when measuring blood brain barrier disruption and resulting brain inflammation in old vs. young animals (**Data not shown**). This allows drawing a clear parallelism between these two compartments, the gut and the brain, which further points at the importance of the gut-brain axis in neurodegenerative diseases, like PD.

The ability of iron to polarize immune cells towards a pro-inflammatory state led us to assess if an increased iron level was present in the intestine of old animals when compared to young mice. As shown in **Figure 4.4**, this was indeed the case.

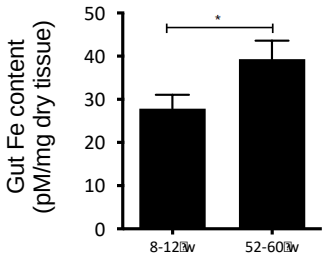

Figure 4.4 Increased iron accumulation in the intestine of old mice in relation to young animals. The results were expressed as mean ± standard deviation (n=10-15 mice per group). Significance has been observed according to t-test.

4.2 Disruption of Iron metabolism During Aging

Changes in intestinal iron absorption, storage and export have been observed in the gut of aged mice, which present an altered expression of iron importers, like the divalent metal transporter 1 (DMT-1) and Transferrin receptor protein 1 (TfR-1), of the intracellular storage Ferritin heavy chain (FtH), and of the iron exporter Ferroportin (FPN). Moreover, since most iron is contained within the protoporphyrin molecule of heme, the expression of the heme-degrading enzyme heme oxygenase (HO-1) was expected as potentially altered and therefore analyzed by qRT-PCR.

When gut samples were collected from 8-12-week-old mice and compared to 52-60-week-old animals, significant differences were observed only for FtH and FPN, as shown in **Figure 4.5**.

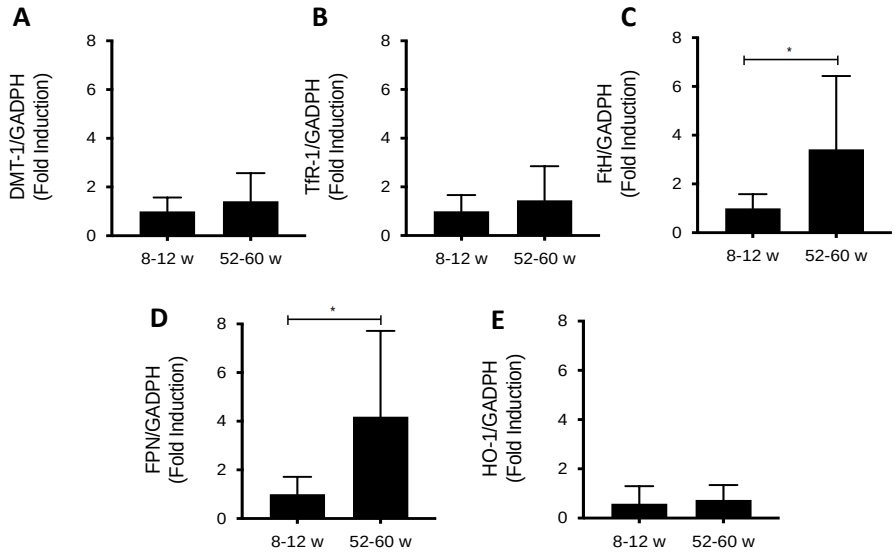

Figure 4.5 Expression of (A) DMT-1, (B) TfR-1 (C) FtH, (D) FPN, and (E) HO-1 was analyzed by qRT-PCR. Expression of GADPH was used as loading control. The results were normalized to GADPH and expressed as fold induction in relation to young animals, used as controls, ± standard deviation (n=10-15 number of mice). Significance has been observed only for FtH and FPN, according to t-test.

The reactivity of iron relies on its ability to participate to the Fenton chemistry and promote the generation of oxidative stress. This was assessed *in vitro* by stimulating intestinal Caco-2 cells with Iron, and then measuring the production of total and mitochondrial radical oxygen species ROS. Increased ROS have been observed only measuring total oxidative stress. No mitochondrial ROS was measured upon the indicated treatment (**Figure 4.6**).

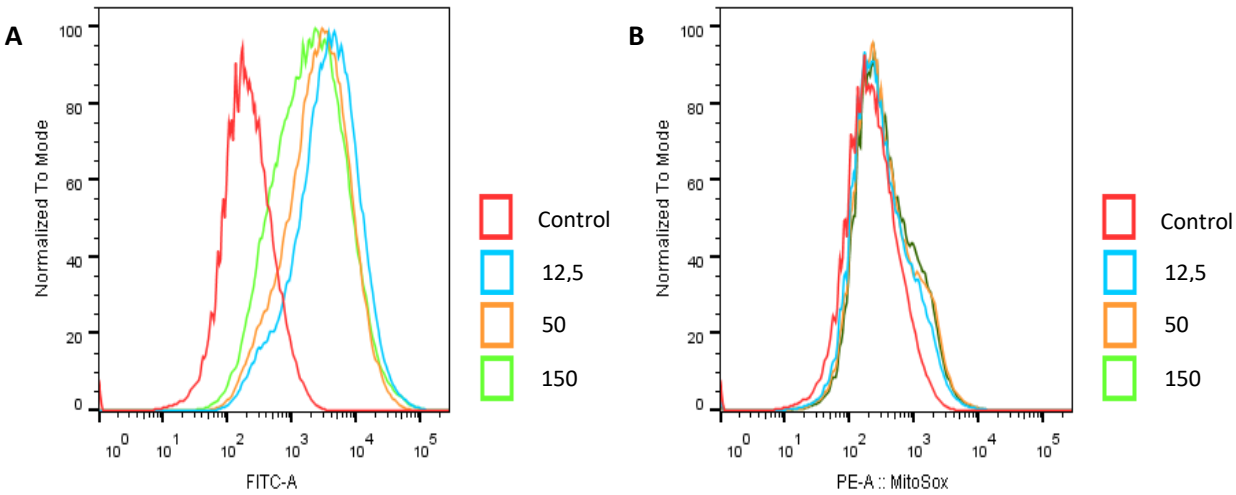

Figure 4.6 (A) Total ROS generation measured by using CM-H2DCFDA, and (B) mitochondrial ROS production assessed by using a mitochondrial specific ROS probe, namely MitoSOX

We then assessed whether an increased oxidative stress might be caused *in vivo* by a reduced expression of antioxidant genes, which could turn the gut more susceptible to the oxidative action of iron. Although no difference was found in the expression of glutathione peroxidase catalase (**Data not shown**) and superoxide dismutase (SOD) in young vs. old mice, this latter shown in **Figure 4.7**, no experiment was conducted to assess if the activity of these enzymes changes during aging.

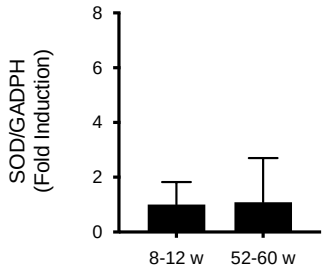

Figure 4.7 The expression of SOD was analyzed by qRT-PCR in indicated samples. Expression of GADPH was used as loading control. The results were normalized to GADPH and expressed as fold induction in relation to young animals, used as controls, ± standard deviation (n=10-15 number of mice).

4.3 Calprotectin expression during aging

The expression of calprotectin (CP) increases with inflammation. Thus, the expectation was to observe higher levels of this protein in the gut of aged mice when compared to young animals, an hypothesis confirmed by qRT-PCR (**Figure 4.8**).

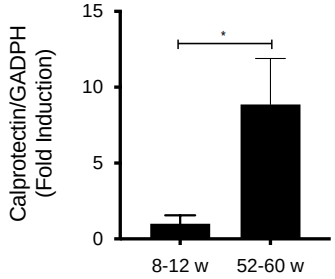

Figure 4.8 The expression of calprotectin was analyzed by qRT-PCR in indicated samples. Expression of GADPH was used as loading control. The results were normalized to GADPH and expressed as fold induction in relation to young animals, used as controls, ± standard deviation (n=10-15 number of mice). Significance has been observed according to t-test.

CP is known to act as heme/iron scavenger. Thus, exogenous CP administration, via intraperitoneal injection of purified protein, was expected to reduce iron accumulation in the gut. Indeed, low levels of iron were observed in the gut of old animals injected with CP when compared to unmanipulated 52-60-week-old mice (**Figure 4.9**).

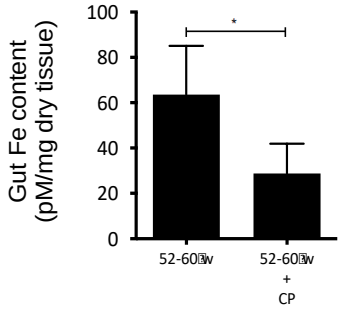

Figure 4.9 Decreased iron accumulation in the intestine of old mice treated or not with calprotectin (administered i.p., 15mg/kg, 5 injections every day). The results were expressed as mean ± standard deviation (n=5 mice per group). Significance has been observed, according to t-test.

The association between CP and iron was further demonstrated by ELISA, when measuring the levels of this protein in the peripheral blood of young mice treated with heme. Increased CP was found in 52-60-week-old mice in relation to 8-12 week-old animals. Injection of heme raised the levels of CP in young but not old mice, possibly due to higher iron level in the gut presented by these animals (**Figure 4.10**). This hypothesis also correlates with the reduced expression of iron importers in the intestine of old mice injected with heme (**Data not shown**), as to prevent further iron uptake.

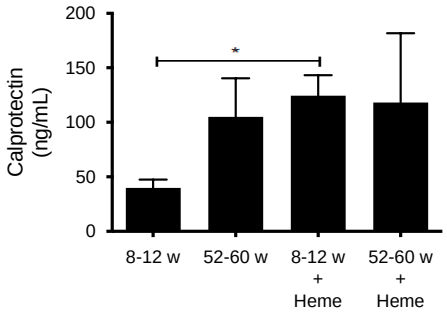

Figure 4.10 Increased level of CP in the sera of young and old mice treated or not with heme (administered i.p., 15mg/kg, 3 injections every day). The results were expressed as mean ± standard deviation (n=5 mice per group). Significance has been observed when comparing young vs. old and young mice injected or not with heme, according to t-test.

Similar results were also observed when measuring the expression of CP in the gut by qRT-PCR of young animals treated or not with heme (**Figure 4.11**). Although CP acts as heme/iron scavenger, repetitive injecti ons of heme might simply saturate its binding capacity, thus possibly explaining an increased in inflammation and, subsequent CP levels, upon intraperitoneal heme administration.

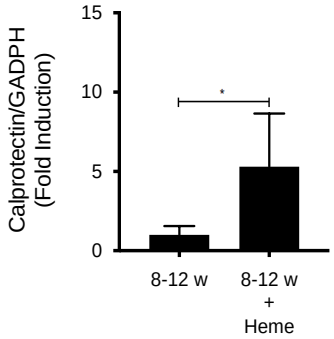

Figure 4.11 The expression of calprotectin was analyzed by qRT-PCR in young mice treated or not with heme (administered i.p., 15mg/kg, 3 injections every day). Expression of GADPH was used as loading control. The results were normalized to GADPH and expressed as fold induction in relation to young animals, used as controls, ± standard deviation (n=10-15 number of mice). Significance has been observed, according to t-test.

Injections of heme have been carried out to boost inflammation. As occurring during aging, the activation of the immune response, from the gut spreads to the brain, priming it to develop neuroinflammation and neurodegeneration. This is coupled with the disruption of iron metabolism, which was assessed in the gut and shown in **Figure 4.12**. Significant differences in relation to FtH and FPN have been observed in heme-treated young animals.

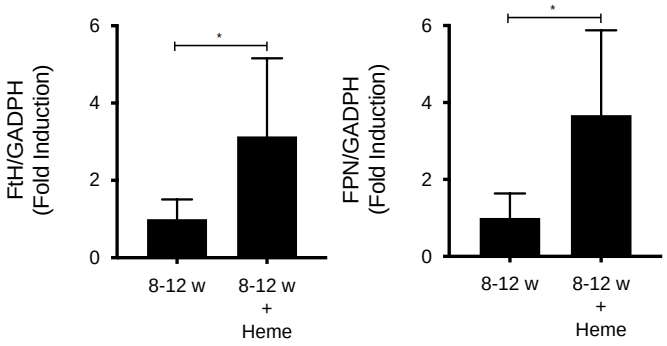

Figure 4.12 The expression of FtH and FPN were analyzed by qRT-PCR in young mice treated or not with heme (administered i.p., 15mg/kg, 3 injections every day). Expression of GADPH was used as loading control. The results were normalized to GADPH and expressed as fold induction in relation to young animals, used as controls, ± standard deviation (n=10-15 number of mice). Significance has been observed, according to t-test.

The importance of these findings relates to other projects conducted in the laboratory, demonstrating the association between an increased iron accumulation in the gut and the enhanced susceptibility to neurodegenerative diseases, like PD. As shown below, old mice exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), used as pharmacological model of PD, develop exacerbated locomotor dysfunction when compared to young animals (**Figure 4.13**). Whether this is due to the phenotype described so far seems to be the case, since increased gut iron accumulation, disruption of epithelial barrier and intestinal inflammation have been identified as important players in increasing PD incidence and severity.

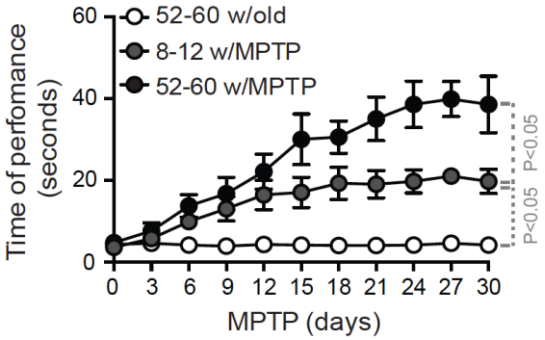

Figure 4.13 Exacerbated locomotor dysfunction measured as time or performance of mice placed up-side-down on a vertical pole, i.e. counting how many seconds they take to descend the pole upon injections of MPTP (i.p., 15mg/kg, 3 injections in total, 1 injection every 2 hours). n=10-15 mice per group. Significance has been observed, according to t-test.

This correlates with an increased level of CP in peripheral blood of MPTP-treated mice in relation to unmanipulated controls (**Figure 4.14**). Interestingly, the extent of CP increase is higher in young mice induced with PD when compared to older animals treated with MPTP. Whether we can justify this finding with the notion that the gut of these latter is already inflamed and therefore subjected to a lower increase in CP levels may be the case. Nevertheless, significant differences have been observed in all groups analyzed.

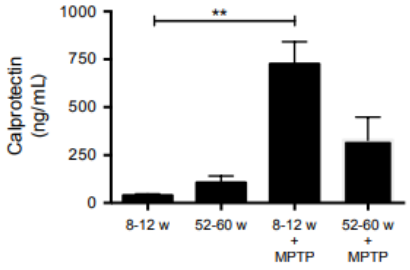

Figure 4.14 Increased level of CP in the sera of young and old mice treated or not with MPTP (administered i.p., 15mg/kg, 3 injections every 2h). The results were expressed as mean ± standard deviation (n=5 mice per group). Significance has been observed, according to t-test.

The important role of iron in PD progression stands out by the successful treatment of PD patients with iron chelation therapy. As mentioned in the introduction section, deferiprone is currently in Phase II clinical trial for PD. In mice, this treatment reduced the accumulation of iron in the gut, as demonstrated by the diminished expression of FtH in this compartment 15 and 30 days after deferiprone administration (**Figure 4.15**).

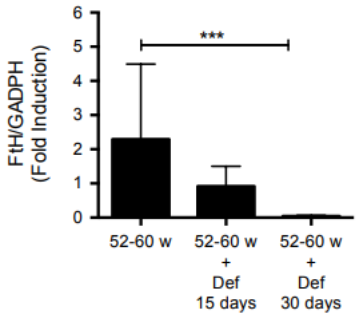

Figure 4.15 The expression of FtH was analyzed by qRT-PCR in old mice treated or not with deferiprone (Def.), for 15 or 30 days (i.p., 15mg/kg, every day). Expression of GADPH was used as loading control. The results were normalized to GADPH and expressed as fold induction in relation to young animals, used as controls, ± standard deviation (n=10 mice per group). Significance has been observed, according to t-test.

By reducing iron, deferiprone decreases inflammation and, subsequently, CP levels as measured after a treatment of 15 days. However, further experiments are needed to better understand why low iron also increases CP, as shown in **Figure 4.16**. Interestingly, this reflects the results obtained in different project, pointing at an increase efficacy of deferiprone when administered for shorter periods of time.

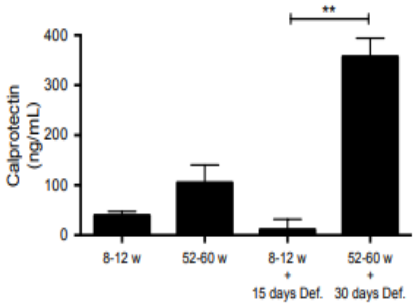

Figure 4.16 Increased level of CP in the sera of young or old mice treated or not with heme (administered i.p., 15mg/kg, 3 injections every day). The results were expressed as mean ± standard deviation (n=5 mice per group). Significance has been observed, according to t-test.

Whether CP could be used as biomarker for gut inflammation-driven PD development was also assessed in another model of PD, based on the injections of purified α-syn.

Several reports in the literature showed that α-syn can spread from the gut to the brain, priming this organ to neuroinflammation and neurodegeneration.

Thus, with the intent to reveal the efficacy of CP as prognostic marker for PD onset, our expectation was to detect an increased level of this protein in mice previously exposed to α-syn, as confirmed in **Figure 4.17**. Contrarily, heme administration prior α-syn injections reduced the release of CP into circulation. However, further experiments will be required to better understand this phenomenon.

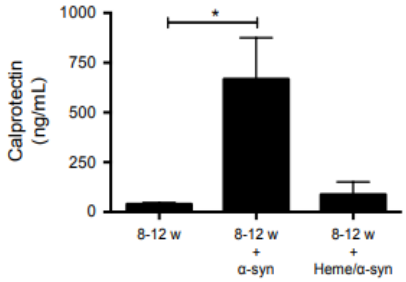

Figure 4.17 Increased level of CP in the sera of young mice treated or not with α-syn and/or heme (administered i.p., 10 μg of α-syn por mice and 15mg/kg of heme, 5 injections every 24h). The results were expressed as mean ± standard deviation (n=5 mice per group). Significance has been observed, according to the t-test.

Our last set of experiments further strengthen the potential use of CP as biomarker for iron-driven gut inflammation and prognostic tool for PD onset. This was already demonstrated by injecting purified CP into mice and measure gut iron accumulation. We demonstrated that exogenous CP administration in old mice reduced the levels of iron in the gut in relation to unmanipulated animals, possibly due to the ability of CP to act as iron scavenger. Similar beneficial effects were observed when measuring the extent of gut epithelial disruption upon exogenous CP administration into old mice. The decrease in gut permeability shown in the graph below allows us to consider calprotectin a potential biomarker to be used as prognostic tool of gut inflammation-driven PD onset (**Figure 4.18).**

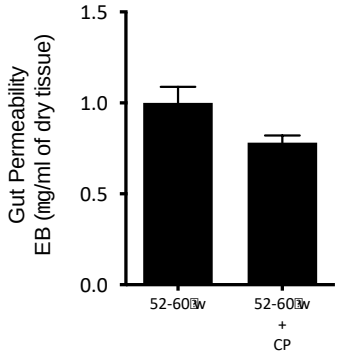

Figure 4.18 Reduced iron accumulation in the intestine of old mice treated or not with calprotectin (administered i.p., 15mg/kg, 5 injections every 24h). The results were expressed as mean ± standard deviation (n=5 mice per group).

5. Discussion

The aim of this study was to correlate the levels of calprotectin to the accumulation of iron in the gut and the resulting inflammation in this compartment, which underlie the development of PD. Our results revealed that CP may represent a biomarker for intestinal dysfunction, which leading to an increased permeability of gut epithelium, could also be used to predict PD onset. Many evidence demonstrate that PD is a pathology that start first in the gut, where α-syn aggregates have also been found (Kim et al., 2019).

Since PD is an age-related disorder (Hindle, 2010), comparisons have been established *in vivo*, by assessing intestinal iron accumulation, gut inflammation, and the levels of calprotectin in young (8-10 weeks old) vs. (52-60 weeks old) mice. Significant differences have been observed in the groups of animals analyzed, as an increase in all parameters evaluated has been found. Thus, our findings fully corroborate the data already published in the literature (Milan-Mattos et al., 2019)(Parrish, 2017)(Ferrucci et al., 2010).

The interplay existing between iron metabolism and inflammation, culminating by one regulating the other, led us to demonstrate that the expression of important genes devoted to maintaining iron homeostasis are impaired also in the gut of aged mice. In particular, the levels of iron importers, like DMT-1 and TfR-1, iron storage gene, like FtH, and the iron exporter, FPN, were quantified. An increase in their expression was observed in the gut of old mice when compared to young. These findings are consistent with the accumulation of iron in these animals, which then influences the absorption, storage and release of this metal from the gut with advancing age.

It is known that the deleterious effects associated with the accumulation of iron in tissues relies on its ability to participate to the Fenton chemistry and generate highly reactive hydroxyl radicals that then damage lipids, proteins and nucleic acids. The gut is no exception. However, the up-regulation of the stress protein HO-1, which is usually indicative of iron-driven oxidative stress and considered a sensitive marker of radical oxygen species (ROS) formation, was not observed in old animals when compared to young. Nevertheless, further experiments would be required to assess the enzymatic activity of HO-1, which can be impaired despite similar levels of gene expression.

In vitro, the stimulation of Caco-2 cells with heme or iron also showed an increased ROS. However, no increase was observed when using a specific mitochondrial probe, indicating that the iron-driven oxidative stress in these cells does not have mitochondria origins. Whether enhanced ROS could be caused also by a decreased expression of antioxidant genes, such as glutathione peroxidase, superoxide dismutase (SOD), catalase, etc., might be the case. However, when assessed in the gut of young vs. old mice, no differences in the expression of aforementioned genes was detected. Similar to previous experiments, additional measurements would need to be carried out to assess the enzymatic activity of these proteins during aging, since differences in their functionality might not reflect changes in their expression.

Recent studies clearly demonstrate the importance of calprotectin in predicting the extent of gut inflammation (Kennedy et al., 2019). The levels of this protein increase in presence of an activated immune response, when measured in the gut and circulation. Thus, the sub-acute chronic inflammatory phenotype developed in both compartments, with advancing, age results in an enhanced calprotectin release in the intestine and bloodstream of older mice. Interestingly, the association between calprotectin and iron has also been reported, with calprotectin being described as an iron scavenger (Nakashige et al., 2015). This notion was confirmed when purified calprotectin was injected into old animals, which presented a reduced iron accumulation in the gut in relation to unmanipulated animals. This led us to

assess whether an increase in calprotectin could be detected in mice injected with heme. This was used in these experiments as vehicle to deliver iron, as shown by the higher expression of analyzed FtH and FPN in response to this treatment. Higher levels of calprotectin were observed in young mice injected with heme. However, no further increase was found in old animals, exposed to the same treatment, possibly due to the already present iron accumulation and the extent of calprotectin increase, observed even at basal level.

Overall, the data obtained might contribute to further understand the pathogenesis of PD, characterized by increased iron accumulation and pro-inflammatory profile, these being observed in both gut and brain. When calprotectin was measured in mice treated with a pharmacological compound capable to trigger PD, increased levels were observed in both young and old animals developing the pathology. However, higher levels of calprotectin were found in PD-induced young mice when compared to old. Already in Phase II clinical trials, iron chelation therapy with deferiprone was proven to be very effective in the treatment of PD, since capable to diminish iron accumulation and subsequent neurodegeneration. When assessed if deferiprone was able to diminish the accumulation of iron also in the gut of old animals, treated for 15 or 30 days, the results obtained demonstrated that this was indeed the case. This was also confirmed by the reduced expression of the intracellular iron storage, FtH. However, while these data correlated with a reduced level of calprotectin after 15 days of treatment, longer exposure to deferiprone significantly increased the release of this protein, presumably due to the occurrence of side inflammatory effects that would need to be investigated.

The use of calprotectin as biomarker for gut inflammation-driven PD development was also assessed in an α-syn injected model of PD. Its levels were enhanced upon α-syn intraperitoneal administration, possibly due to the local inflammation caused by this treatment. However, reduced calprotectin was observed when mice were also injected with heme. Whether this is due to the synergistic action of two heme scavengers, i.e. the endogenous calprotectin and the exogenously administered α-syn, could be the case, as these two proteins might join efforts to prevent the inflammatory phenotype triggered by heme.

To further strengthen the hypothesis that CP is capable to serve as biomarker for iron-driven gut inflammation, which then leads to PD onset, exogenous administration of purified CP was carried out in older animals, where improvement of gut permeability has been observed in response to this treatment. This result strongly corroborated a role of calprotectin as iron scavenger in the gut. In fact, in the anaerobic environment of the gut, calprotectin injections result in the binding of Fe(II) to this protein, thus which prevents the Fenton reactivity of this metal and the subsequent disruption of gut epithelium. In agreement, we believe that by restoring intestinal function, exogenous administration of calprotectin might also inhibit the inflammatory phenotype that might ultimately impact on the brain.

6. Conclusion

This project provides new insights into the importance of Calprotectin in the development of Parkinson's disease. We conclude that inflammation is correlate with increased iron accumulation in the gut, highlighting the direct correlation of aging with iron levels. Iron accumulation is associated with the formation of reactive oxygen species; however, our results of gene expression don't show ROS formation in old animals when compared to young ones. It is important to remember that it has been reported that the presence of iron prone to α-syn aggregation.

CP acts as a proinflammatory cytokine that is released in situations of inflammation and therefore may serve as a biomarker of intestinal inflammation caused by iron. Since the gut has an anaerobic environment and CP binds only the reduced and most reactive form of iron, ie Fe (II), making it a perfect place for this protein to function. In addition, CP levels allow you to expect intestinal inflammation, which in turn may help to prevent the severity of PD. Our experiments have allowed us to observe the increase in Calprotectin levels in the presence of high levels of inflammation, as well as in experiments that mimicked PD experiments. Indeed, exogenous CP administration in old mice decreases the levels of iron in the gut and the extent of gut epithelial disruption, factors of several inflammatory disorders, like PD. Restoring adequate levels of calprotectin may reduce the incidence of PD and / or slow the progression of PD, as this protein acts as an iron scavenger.

New insights into the relevance of CP, as a mediator of inflammation and iron scavenger, showing a possible protective effect against α-syn aggregation, as contributions to add knowledge to an underdeveloped field.

7. References

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